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Regulation of the pentose phosphate cycle

Cofactor that controls the inhibition of glucose-6-phosphate dehydrogenase by NADPH in rat liver

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A cofactor of M_r 10^4, characterized as a polypeptide, was found to co-operate with GSSG to prevent the inhibition of glucose-6-phosphate dehydrogenase by NADPH, in order to ensure the operation of the oxidative phase of the pentose phosphate pathway, in rat liver [Eggleston & Krebs (1974) Biochem. J. **138**, 425–435; Rodriguez-Segade, Carrion & Freire (1979) Biochem. Biophys. Res. Commun. **89**, 148–154]. This cofactor has now been partially purified by ion-exchange chromatography and molecular gel filtration, and characterized as a protein of M_r 10^5. The lighter cofactor reported previously was apparently the result of proteolytic activity generated during the tissue homogenization. The heavier cofactor was unstable, and its amount increased in livers of rats fed on carbohydrate-rich diet. Since the purified cofactor contained no glutathione reductase activity, the involvement of this enzyme in the deinhibitory mechanism of glucose-6-phosphate dehydrogenase by NADPH should be ruled out.

INTRODUCTION

The oxidative phase of the pentose phosphate cycle is predominantly responsible for the production of NADPH for the synthesis of fatty acids and other molecules in animal tissues. Strongest control of this pathway occurs under conditions where lipogenic activity is enhanced. 'Coarse control', consisting of adaptative variation of the amount of enzymes present, was described by Tepperman & Tepperman (1958), Fitch *et al.* (1959*a*,*b*), Fitch & Chaikoff (1960) and Winberry & Holten (1977), who found that lipogenesis in rats fed with excess of carbohydrate was accompanied by 5–10-fold increases in the amounts of both glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the liver. 'Fine control' of these enzymes has been shown to depend on the cytosolic [$NADP^+$]/[NADPH] ratio (Kather *et al.*, 1972; Sapag-Hagar *et al.*, 1973), NADPH being a potent inhibitor of both G6PDH and 6PGDH, competitive with $NADP^+$. Ratios of [$NADP^+$]/[NADPH] within the range of 9:1 cause total inhibition of purified rat liver G6PDH (Eggleston & Krebs, 1974). Since the ratio of these nucleotides in rat liver cytoplasm is of the order of 100:1 (Veech, 1968; Veech *et al.*, 1969), it could be inferred that the oxidative phase of the pentose cycle would not operate *in vivo*. In an attempt to resolve this paradox, Eggleston & Krebs (1974) found that, in rat liver homogenates prepared under hypo-osmotic conditions, GSSG and a cofactor of M_r less than 15000 co-operated to overcome the inhibition of G6PDH by NADPH. A similar effect of GSSG and cofactor was described by Rodriguez-Segade *et al.* (1978) in mussel hepatopancreas. These authors have characterized this cofactor, in rat liver and hepatopancreas hypo-osmotic homogenates, as a polypeptide of M_r 10^4 and 1.5×10^4 respectively (Rodriguez-Segade *et al.*, 1979).

While attempting to purify the rat liver cofactor to characterize its mechanism of action in preventing G6PDH inhibition, we found that the cofactor activity present in unfractionated iso-osmotic homogenates was completely absent from the components of M_r less than 5×10^4.

We accordingly undertook a systematic search for cofactor activity in both iso-osmotic and hypo-osmotic homogenates. The partial purification and properties of a cofactor of M_r 10^5, as well as the observation that the lighter cofactor previously reported is an artefact, are described in this paper.

MATERIALS AND METHODS

Chemicals

NADPH, $NADP^+$, GSSG, glucose 6-phosphate, trypsin, trypsin inhibitor, 6-amino-n-hexanoic acid, phenylmethanesulphonyl fluoride, DEAE-cellulose and CM-cellulose were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and Sephadex G-150 and G-25 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Other chemicals used, including standards for M_r determinations, were obtained from the same sources as previously (Rodriguez-Segade *et al.*, 1979).

Animals

Wistar rats of about 200–300 g were either fed on a standard laboratory diet or starved for 2 days before being kept in darkness for 3 days while being fed on a diet containing 66% saccharose and 1% (w/w) fat.

Fractionation of rat liver homogenates

Rat liver homogenates were prepared in 4 vol. of EDTA/phosphate buffer (50 mM-potassium phosphate, 2 mM-EDTA, pH 7.4). In some experiments 0.25 M-sucrose or proteinase inhibitors (1 mM-amino-n-hexanoic acid plus 1 mM-phenylmethanesulphonyl fluoride) were included. After centrifugation of the homogenates at 15000 *g* for 15 min, the supernatants were centrifuged at

Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 6PGDH, 6-phosphogluconate dehydrogenase (EC 1.1.1.44).

100000 *g* for 1 h. The supernatants were either filtered through Amicon Centriflo membrane cones (224-CF-50A; Amicon N.V., Oosterhout, Holland), or passed through DEAE-cellulose columns (9 cm × 2 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.4. The break-through peak, which contained no G6PDH activity, was chromatographed on a Sephadex G-150 column (50 cm × 2 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.4.

Purification of cofactor

Rat liver iso-osmotic 100000 *g* supernatants were fractionated by DEAE-cellulose chromatography as indicated above. The void-volume fractions were passed through a column (72 cm × 2.5 cm) of Sephadex G-25 (coarse grade) equilibrated with 20 mM-potassium phosphate buffer, pH 6.1, and the protein peak was loaded on a CM-cellulose column (3 cm × 3 cm) equilibrated with the same buffer. The column was eluted with the equilibration buffer, followed by a linear gradient (50 ml + 50 ml) of 0–0.5 M-KCl in this buffer. The cofactor-containing fractions were adjusted to pH 5 with acetic acid and loaded on a CM-cellulose column (7 cm × 1.5 cm) equilibrated with 20 mM-potassium phosphate/acetic acid buffer, pH 5. After washing with the equilibration buffer, the column was eluted with a linear gradient (70 ml + 70 ml) of 0–0.2 M-KCl in this buffer. Fractions with the highest cofactor activity were further chromatographed on a Sephadex G-150 column (35 cm × 2 cm) equilibrated and eluted with 20 mM-potassium phosphate buffer, pH 7.4.

Enzyme assays

Activities of G6PDH and glutathione reductase (EC 1.6.4.2) were determined spectrophotometrically by measuring changes in A_{340} for 8 min at 25 °C.

G6PDH was determined in a reaction mixture (1 ml) containing 60 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 100 μM-$NADPH^+$ and various concentrations of homogenates, in the presence or absence of 6 mM-glucose 6-phosphate. Blanks without glucose 6-phosphate were run simultaneously. One unit of G6PDH activity was defined as the amount of enzyme required to form 1 μmol of NADPH/min with both substrates in excess.

Glutathione reductase activity was assayed in a reaction mixture (1 ml) containing 60 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 20 μM-$NADP^+$, 80 μM-NADPH and various concentrations of extracts in the presence or absence of 0.1 mM-GSSG. One unit of glutathione reductase activity was defined as the amount of enzyme required to form 1 μmol of $NADP^+$/min with both substrates in excess.

Cofactor activity

The reaction mixture (1 ml) contained 60 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 20 μM-$NADP^+$, 80 μM-NADPH, 6 mM-glucose 6-phosphate and various concentrations of tissue homogenates or different concentrations of purified G6PDH and cofactor in the presence or absence of 0.1 mM-GSSG. The cofactor activity was determined by measuring changes in A_{340} at 25 °C and expressed as the percentage of activation exercised against the inhibition of G6PDH by NADPH (Rodriguez-Segade *et al.*, 1978).

Protein determination

Protein was determined by the methods of Lowry *et al.* (1951) and Bradford (1976). Specific activity is expressed as units of enzyme activity per mg of protein.

Purification of glucose-6-phosphate dehydrogenase

Livers were homogenized in 4 vol. of 20 mM-potassium phosphate buffer, pH 6.4, 2 mM-EDTA, 7 mM-2-mercaptoethanol and 10 μM-$NADP^+$. Supernatants after centrifugation at 15000 *g* for 15 min were further centrifuged at 100000 *g* for 1 h. These supernatants were fractionated by the addition of solid $(NH_4)_2SO_4$ to 25% saturation, and precipitated proteins were spun down at 15000 *g* for 20 min and discarded, whereas the supernatant fraction was made 75% saturated with $(NH_4)_2SO_4$. After centrifugation at 15000 *g* for 20 min, the pellet was dialysed and chromatographed on a DEAE-cellulose column (10 cm × 2 cm), equilibrated with the homogenization buffer. The enzyme was eluted with a linear gradient from 20 mM- to 0.6 M-potassium phosphate buffer, pH 6.3. The fractions with highest G6PDH activity were pooled, dialysed against 20 mM-potassium phosphate buffer, pH 7, concentrated over solid sucrose and purified by high-performance ion-exchange chromatography on a DEAE 5-PW column (Waters Associates) with a Kontron h.p.l.c. system model 600. The column was eluted at room temperature with 20 mM-potassium phosphate buffer, pH 7, for 10 min, and then with a gradient of 0.02–0.4 M of the same buffer in 45 min. The flow rate was 1 ml/min, and fractions were collected every 1 min at 0 °C. The eluate was monitored at 230 nm,

Effect of trypsin

The effect of trypsin on the cofactor activity was assayed by incubation of 300 μg of purified cofactor with trypsin (12 μg) at room temperature. After 2 h, trypsin inhibitor (12 μg) was added and the cofactor activity determined in 50 μl samples of the incubation mixture.

Polyacrylamide-gel electrophoresis

Polyacrylamide gels (0.6 cm × 9 cm) were prepared and electrophoresis was carried out by the procedure of Davis (1964). Samples of purified cofactor (0.2 mg) were electrophoresed for 4 h at 4 °C. After electrophoresis the gels were stained or cut into 1.5 mm slices and extracted with 0.6 ml of 50 mM-Tris/HCl buffer, pH 7.4, for 1 h at 4 °C. The cofactor activity was assayed in 50 μl samples of each slice extract.

RESULTS AND DISCUSSION

Effect of carbohydrate-rich diet on the mechanism preventing the inhibition of G6PDH by NADPH

To study the effect of lipogenic conditions on the deinhibition of G6PDH by the cofactor–GSSG system, livers of rats fed on normal and carbohydrate-rich diets were used. As shown in Table 1, the deinhibitory effect in homogenates from rats fed on carbohydrate-rich diet was 3 times that in those from rats fed on normal diet. This increase is probably not caused by a rise of endogenous GSSG concentrations, because a similar increase was observed in homogenates that had been dialysed or chromatographed on Sephadex G-150. It also cannot be attributed to changes in the activities of G6PDH or glutathione reductase, because in our assay

Table 1. Cofactor activity in unfractionated and ultrafiltered iso-osmotic and hypo-osmotic liver homogenates from rats fed on carbohydrate-rich or normal diets

Cofactor activity (see the Materials and methods section) in unfractionated homogenates was determined in 5 μl samples (110 μg of protein). Activity in ultrafiltrates was assayed in 5 μl samples (85 μg of protein), by adding 22 μg of G6PDH partially purified by DEAE-cellulose chromatography (specific activity 98 units/mg). Results are means ± S.D. for the numbers of determinations in parentheses.

	Cofactor activity in rat liver homogenates (%)			
	Hypo-osmotic		Iso-osmotic	
Diet	Unfractionated	Ultrafiltered	Unfractionated	Ultrafiltered
High-carbohydrate	72 ± 13 (10)	32 ± 7 (4)	75 ± 18 (10)	0 (10)
Normal	23 ± 4 (6)	20 ± 9 (4)	25 ± 3 (6)	0 (10)

conditions the G6PDH activity was kept constant, and no appreciable variation in the glutathione reductase activity was observed. It therefore seems that lipogenic conditions induce an increase in the concentrations of cofactor in order to prevent the inhibition of G6PDH and, consequently, to provide a higher concentration of NADPH required in that physiological state. This supports the hypothesis that variation in cofactor concentration might constitute a mechanism for the control of the G6PDH activity, additional to that exerted by the increase in the activity of this enzyme in response to a diet containing excess of carbohydrates (Tepperman & Tepperman, 1958).

Size fractionation of rat liver homogenates

When iso-osmotic homogenates obtained from livers of rats fed on lipogenic diet were passed through Amicon 224 CF-50A cones, it was found that the filtrates (containing cell components of M_r lower than 5×10^4) gave no protection of G6PDH against inhibition by NADPH, whereas the deinhibitory effect was observed in the corresponding filtrates of hypo-osmotic homogenates. Experiments carried out with both iso- and hypo-osmotic homogenates obtained from rats fed on normal diet confirmed this discrepancy (Table 1).

To explain this discrepancy, we fractionated rat liver homogenates by DEAE-cellulose chromatography in 50 mM-potassium phosphate buffer, pH 7.4. Under these conditions G6PDH was completely retained on the column, whereas the deinhibitory activity, in both iso- and hypo-osmotic homogenates, was eluted in the void-volume fractions (results not shown). Gel filtration of the latter fractions on Sephadex G-150 (Fig. 1*a*) showed that in the iso-osmotic homogenates the deinhibitory effect on G6PDH inhibition was found exclusively in the fractions whose components had M_r of the order of 10^5. In the hypo-osmotic homogenates there was also a minor deinhibitory activity corresponding to components of M_r about 10^4. This latter presumably corresponds to the cofactor reported by Eggleston & Krebs (1974) and characterized as a polypeptide (Rodriguez-Segade *et al.*, 1979). According to our results, it seems that this polypeptide may have been an artefact produced during the hypo-osmotic homogenization, probably by proteolytic degradation. To test this hypothesis, hypo-osmotic homogenates prepared, in the presence of proteinase inhibitors, from livers of rats fed

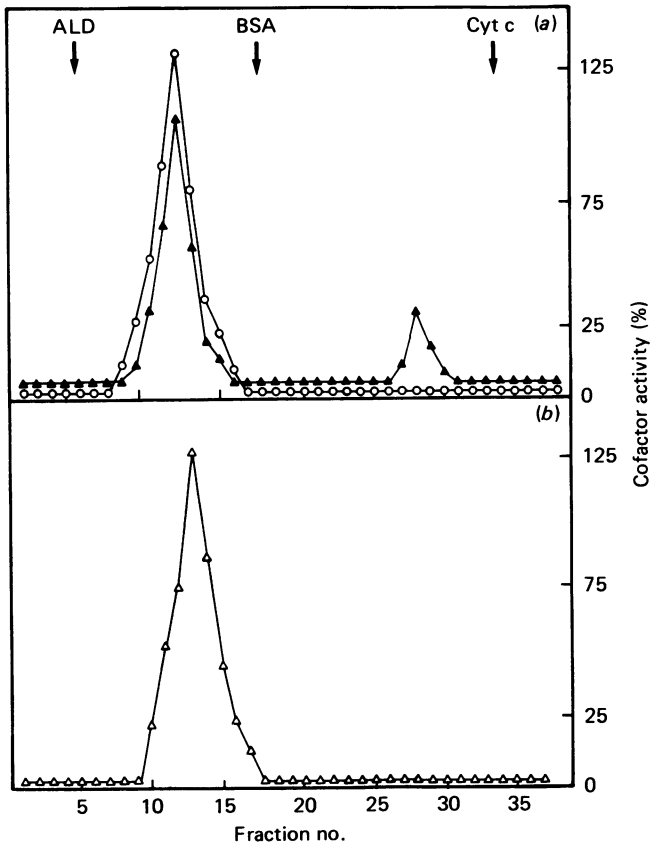

Fig. 1. Sephadex G-150 chromatography of rat liver homogenates

Samples (25 ml; about 500 mg) of iso-osmotic and hypo-osmotic homogenates (*a*) and hypo-osmotic homogenates in the presence of proteinase inhibitors (*b*) were chromatographed through a Sephadex G-150 column (50 cm × 2 cm), equilibrated and eluted with 50 mM-potassium phosphate buffer, pH 7.4. Fractions (5.5 ml) were collected and activities of iso-osmotic (○) and hypo-osmotic cofactor in the presence (△) or absence (▲) of proteinase inhibitors were determined in 50 μl samples of each fraction by adding 22 μg of G6PDH purified by DEAE-cellulose chromatography as described in the Materials and methods section. The arrows show the elution volumes of the markers (ALD, aldolase; BSA, bovine serum albumin; Cyt c, cytochrome *c*).

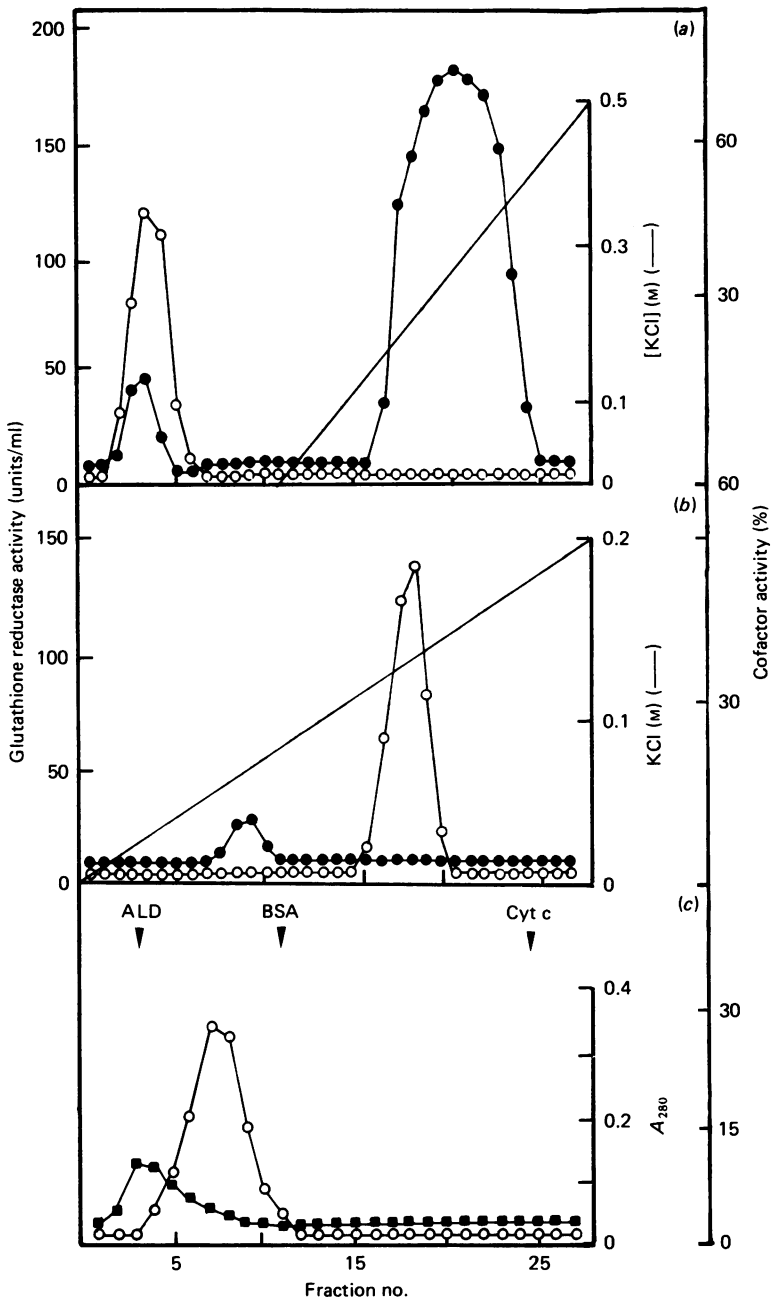

Fig. 2. Purification of the cofactor

Details of the purification procedure are given in the Materials and methods section. Cofactor-containing fractions from the Sephadex G-25 column (about 500 mg) were chromatographed on a CM-cellulose column at pH 6.1 (*a*); 5 ml fractions were collected, and the activities of cofactor (○) and glutathione reductase (●) were assayed simultaneously in 10 μl samples of each fraction as indicated in Fig. 1. Fractions with highest cofactor activity were chromatographed on a CM-cellulose column equilibrated at pH 5 (*b*); 50 μl samples of each fraction (5 ml) were used to assay cofactor and glutathione reductase activities as above. From this column the two fractions with highest cofactor activity were chromatographed by Sephadex G-150 (*c*), with markers as in Fig. 1. The cofactor activity was assayed in 50 μl samples of each fraction (3 ml). Protein was monitored at 280 nm (■).

on high-carbohydrate diet were stripped of G6PDH activity and subjected to gel filtration through Sephadex G-150. The elution patterns (Fig. 1*b*) indicated that there was no cofactor activity among the lighter components, thus confirming that the cofactor reported previously has been produced by proteolysis in the homogenization process. The artefact nature of the lighter cofactor also seems to be supported by the cofactor activity found in ultrafiltered hypo-osmotic homogenates obtained from livers of rats fed on lipogenic or normal diets (Table 1). Thus, although amounts of cofactor are increased by about 150% by a lipogenic diet, the increase in the ultrafilterable cofactor activity was only about 50%.

Purification and properties of the cofactor

In view of these results, we proceeded to purify the cofactor from iso-osmotic homogenates from livers of rats fed on a lipogenic diet. After fractionation of those

Table 2. Purification of the cofactor

Cofactor activity in homogenate was determined in 110 μg samples of protein as indicated in Table 1. Activity at each purification steps was assayed in samples of 85 μg (DEAE-cellulose), 25 μg (CM-cellulose pH 6.1), 19 μg (CM-cellulose pH 5) and 6 μg (Sephadex G-150); G6PDH was added as indicated in Fig. 1.

Step	Volume (ml)	Protein (mg/ml)	Cofactor activity (%)
Homogenate	20	22	75
DEAE-cellulose	20	17	100
CM-cellulose, pH 6.1	20	2.6	55
CM-celulose, pH 5	10	0.39	53
Sephadex G-150	5	0.12	30

homogenates by DEAE-cellulose in 50 mM-potassium phosphate buffer, pH 7.5, fractions containing the cofactor were further purified by chromatography on CM-cellulose and Sephadex G-150 (Fig. 2). For reasons discussed below, particular attention was paid to the presence of glutathione reductase activity, which was completely separated from the cofactor activity as shown in Fig. 2. Table 2 summarizes the different steps in the purification procedure. A significant loss of cofactor activity occurred during purification, thus making prolonged manipulations unsatisfactory. Attempts to purify the cofactor by high-performance ion-exchange chromatography were unsuccessful.

Electrophoretic analysis of purified cofactor showed the presence of a major band, with relative electrophoretic mobility 0.27, together with two less mobile minor bands. In extracts of the slices from gels run in parallel (see the Materials and methods section), the cofactor remained active, and it was associated with the major band (results not shown). Therefore cofactor activity seems apparently to be exerted by a single protein. The purified cofactor lost all activity when was incubated with trypsin for 2 h at 25 °C, confirming its protein nature.

Although partially purified G6PDH was normally used in cofactor assays, no significant difference in the cofactor activity was observed when G6PDH (specific activity 4000 units/mg) purified by h.p.l.c. (see Fig. 3) and cofactor from the various stages of purification were used.

Dialysis of is-osmotic homogenates of livers from rats fed on a lipogenic diet against water at 4 °C showed that the cofactor activity was not lost to any considerable extent within the first 3 h, whereas hypo-osmotic homogenates lost about half their cofactor activity after 3–4 h under these conditions. However, as shown in Table 3, the cofactor in the iso-osmotic homogenates was unstable, especially at 25 °C. Similar instability was observed for cofactor from various stages of the purification process.

Glutathione reductase activity and deinhibitory effect

Since glutathione reductase, by consuming NADPH and GSSG, could interfere with the deinhibitory assay, we have followed the activity of this enzyme through the purification procedure of the cofactor. As shown in Fig. 2, the glutathione reductase was completely separated from the cofactor activity by CM-cellulose chromatography. Furthermore, the assays of the cofactor stability (Table 3) show that its effect is largely independent of the gluathione reductase activity. The interference of glutathione reductase in the deinhibitory effect has been extensively discussed (Eggleston & Krebs, 1974; Rodriguez-Segade *et al.*, 1978, 1979), and it has even been suggested that in rat liver homogenates this effect

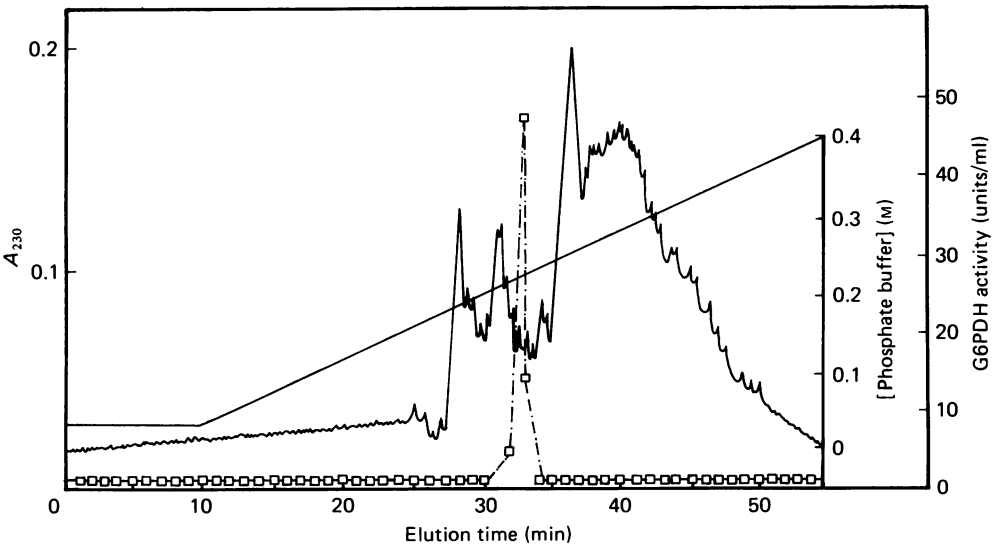

Fig. 3. Purification of G6PDH by high-performance ion-exchange chromatography

A 500 μg sample of partially purified G6PDH, in 100 μl of 20 mM-potassium phosphate buffer, pH 7, was chromatographed on a DEAE 5PW column. The G6PDH activity (□) was eluted with a linear gradient of 0.02–0.4 M-potassium phosphate buffer (shown by straight lines), pH 7, and assayed in 50 μl samples of each fraction (see the Materials and methods section). Protein was monitored at 230 nm (——), with peaks.

Table 3. Stability of cofactor and glutathione reductase activities in iso-osmotic rat liver homogenates

Activities of cofactor and glutathione reductase were simultaneously assayed for each time and temperature in 5 μl samples of the iso-osmotic homogenates (110 μg of protein) as described in the Materials and methods section.

Time (h)	4 °C		25 °C	
	Cofactor activity (%)	Glutathione reductase activity (units/ml)	Cofactor activity (%)	Glutathione reductase activity (units/ml)
0	75	532	75	532
12	50	532	10	425
24	20	485	0	380

could be attributable to the glutathione reductase activity (Levy & Christoff, 1983). Although we have proved that the glutathione reductase did not interfere with the cofactor activity (Rodriguez-Segade *et al.*, 1985), the present results rule out any participation of this enzyme in the deinhibitory mechanism.

We have previously found that the cofactor–GSSG effect also occurs with 6PGDH in rat liver and mussel hepatopancreas (Rodriguez-Segade *et al.*, 1978, 1979). In accordance with this are our recent experiments, which show that rat liver purified cofactor is similarly effective in preventing the inhibition of 6PGDH by NADPH (M. Nogueira, G. Garcia, C. Mejuto & M. Freire, unpublished work). Therefore the deinhibitory mechanism on both dehydrogenases would explain the apparent imbalance in the activities of these enzymes observed by Sapag-Hagar *et al.* (1973). Cofactor and GSSG would become the factors predicted by those authors to overcome the inhibition of G6PDH and 6PGDH by NADPH, in order to make the pentose phosphate cycle operative *in vivo*.

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